



## Independent regulation of adherens and tight junctions by tyrosine phosphorylation in Caco-2 cells

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### Abstract

To study the role of tyrosine phosphorylation in the control of intercellular adhesion of intestinal cells, we have generated several clones of Caco-2 cells that express high levels of pp60v-src only after addition of butyrate. Expression of this oncogene in cells 5 days after confluence induced  $\beta$ -catenin and p120-ctn tyrosine phosphorylation, redistribution of E-cadherin to the cytosol and disassembly of adherens junctions. However, tight junctions of Caco-2 cells at 5 days after confluence were not altered by expression of pp60v-src. Similar results were obtained when Caco-2 cells were incubated with phosphotyrosine phosphatase inhibitor orthovanadate. Although addition of this compound to postconfluent cells disrupt adherens junctions, tight junctions remain unaltered, as determined measuring monolayer permeability to mannitol or hyperphosphorylation of Triton-insoluble occludin. Modifications in tight junction permeability of Caco-2 were only observed at high concentrations of orthovanadate (1 mM). Interestingly, this tyrosine phosphorylation-refractory state was achieved after confluence since early postconfluent cells (day 2) showed a limited but significant response to low doses of orthovanadate. These results suggest that tight junctions of differentiated Caco-2 cells are uncoupled from adherens junctions and are insensitive to regulation by tyrosine phosphorylation. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Tight junction; Adherens junction; pp60v-src; Tyrosine phosphorylation

### 1. Introduction

The primary function of tight junctions (TJ) in epithelial cells is to create a regulated barrier in the extracellular space; furthermore, they are essential for the polarisation of epithelial cells since they create the boundary between the apical and the basolateral domains of the plasma membrane [1]. The integrity of the tight junctions can be regulated by

intracellular transduction pathways; in particular, activation of protein kinase C (PKC) by phorbol-12-myristate-13-acetate (PMA) [2], expression of the product of the v-src oncogene [3] or inhibition of several unidentified phosphotyrosine (Ptyr) phosphatases [4] have been shown to induce a rapid increase of paracellular permeability in MDCK cells. In these cells, the agents mentioned above not only affected TJs but also adherens junctions (AJ) [5,6]. Blocking the function of the main component of AJ (E-cadherin) [7] causes not only the disruption of the AJ but also the opening of TJ [8]. For this reason, it has been generally assumed that both adhesion com-

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plexes are commonly regulated and that the permeability of TJ depends on the integrity of AJ.

The human colon carcinoma cell line Caco-2 represents a widely used *in vitro* system to study intestinal differentiation [9]. After establishing cell contacts, Caco-2 form a well-polarised monolayer showing, at ultrastructural level, tight junctions, a basal nucleus and a well-developed brush-border resembling that of mature small bowel enterocytes or 15th-week foetal colonocytes [9–11]. Moreover, post-confluent Caco-2 cells produce enzymes characteristic of mature enterocytes; some of these markers have only been observed in culture in this cell line [12]. All these properties indicate that differentiated Caco-2 present the most similar phenotype to mature enterocytes that can be obtained *in vitro*.

Our group is interested in the study of regulation of intercellular contacts in intestinal cells. We have observed that TJ and AJ can be modulated by addition of the phorbol ester PMA to HT-29 cell subpopulations [13,14]; similar effects were observed in other intestinal cell lines. However, when we tried to extend this study to well-differentiated Caco-2 cells, the effect was not observed. This result prompted us to further investigate the regulation of TJ and AJ in these cells. In this report we describe that, in contrast to other cell lines, TJ of Caco-2 cells are insensitive to conditions that increase tyrosine phosphorylation, i.e., addition of orthovanadate or expression of pp60v-src.

## 2. Materials and methods

### 2.1. Reagents

PMA, G-418, phenylarsine oxide (PAO), leupeptin, aprotinin and sodium orthovanadate ( $\text{VaO}_4\text{Na}_3$ ) were purchased from Sigma Chemical (St Louis, MO). Protein A-agarose beads and phenylmethylsulfonyl fluoride (PMSF) were from Boehringer (Mannheim, Germany). [ $1\text{-}^{14}\text{C}$ ]Mannitol was from Amersham. The source of the antibodies used in this study was: mouse monoclonal antibodies (mAbs) anti-phosphotyrosine (4G10) and anti-pp60 v-src (EC-10), from Upstate Biotechnology (Lake Placid, NY); mAb anti-E-cadherin (HECD-1) and rabbit polyclonal antibody (pAb) anti-occludin, from

Zymed Laboratories (South San Francisco, CA); mouse mAbs anti- $\beta$ -catenin and anti-p120-cat, from Transduction Laboratories (Lexington, KY).

### 2.2. Cell culture

The cell lines Caco-2 and the two HT-29 subpopulations (HT-29 M6 and HT-29 M3) were supplied by Dr. Zweibaum (INSERM, Villejuif, France). Cells were seeded at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (Caco-2) or 10% (HT-29) foetal bovine serum (FBS) (Gibco, Glasgow, UK) in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Culture media was changed every other day to avoid nutrient depletion. Except for otherwise indicated, experiments with Caco-2 cells were always performed 4–5 days after reaching confluence when cells showed the presence of domes. Fibroblasts 3Y1 and 3Y1 transformed with v-src oncogene were provided by Dr. J. Moscat (CSIC, Madrid, Spain).

### 2.3. Cell transfection

Full length v-src DNA, obtained from ATCC, was cut with *EcoRI* and cloned in the *EcoRI* site of pcDNA3 (Invitrogen, Carlsbad, CA), a vector designed to express in eukaryotic cells cDNAs under the strong cytomegalovirus (CMV) early promoter. Caco-2 cells were transfected with 4  $\mu\text{g}$  of purified plasmid using the lipofectamine protocol (Gibco BRL, Gaithersburg, MD). G-418-resistant clones were isolated after 21 days of selection in DME medium containing 0.8 mg/ml G-418 (Sigma). Positive clones were selected by Western blot with mAb EC-10 that recognises pp60v-src and not pp60c-src.

### 2.4. Monolayer permeability

Cells were cultured onto polycarbonate filters (Transwells, 0.2  $\mu\text{m}$  pore size, Costar, Cambridge, MA) under the conditions described. Permeability of the cell monolayer was determined at the different days after confluence using [ $^{14}\text{C}$ ]mannitol; this compound is not metabolised by the epithelial cells and is not able to go through their junctional system. After adding the [ $^{14}\text{C}$ ]mannitol (0.3  $\mu\text{Ci}$ ) to the upper compartment, the permeability of the monolayer was de-

terminated by counting the radioactivity present in the basal chamber at different times. As control, a transwell where no cells were plated on the filter was used. The flux of [ $^{14}\text{C}$ ]mannitol measured in this control sample was  $2.1 \times 10^4$  cpm/h  $\text{cm}^2$  ( $\pm 0.1$ ); the equilibration between the two chambers was achieved in 2–3 h.

### 2.5. Indirect immunofluorescence

Immunofluorescence staining was performed on cells grown on glass coverslips as described [15]. When indicated, cells were washed for 30 s with CSK buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 1 mM EGTA; 1% Triton X-100, 20  $\mu\text{g}/\text{ml}$  leupeptin; 10  $\mu\text{g}/\text{ml}$  aprotinin, 1 mM PMSF and 0.5 mM  $\text{VaO}_4$ ) before fixation. Cells were incubated for 1 h with mouse mAb anti-E-cadherin HECD-1 (1:400 dilution); after washing, coverslips were incubated with TRITC-labelled anti-mouse IgG (Vector, Burlingame, CA) and visualised in a fluorescence microscope.

### 2.6. Semithin and ultrathin sections

Cell monolayers were rinsed twice with PBS and fixed with 2.5% glutaraldehyde in PBS. Postfixation was performed for 1 h at room temperature with 2%  $\text{OsO}_4$ . Cells were further rinsed twice in PBS for 5 min and dehydrated for 10-min washes in ascending series of graded ethanol (once in 50%, once in 70%, twice in 95% and five times in 100%). Cells were embedded in Spur resin (TAAB Laboratories, Aldermaston, UK). For light microscopy, semithin sections were cut with a LKB ultramicrotome and stained with toluidine blue. For transmission electron microscopy, ultrathin sections of 500–800 Å were placed on uncoated 300-mesh copper grids prior to staining with uranyl acetate (5% in absolute ethanol) and lead citrate and viewed at original magnifications from 5000 to 45000 in a Hitachi H700 transmission electron microscope operated at 75 kV.

### 2.7. Immunoprecipitation

Cell extracts were made in RIPA buffer (20 mM MOPS, pH 7.0; 150 mM NaCl; 1% sodium deoxycholate; 1% Nonidet P-40; 0.1% SDS; 1 mM

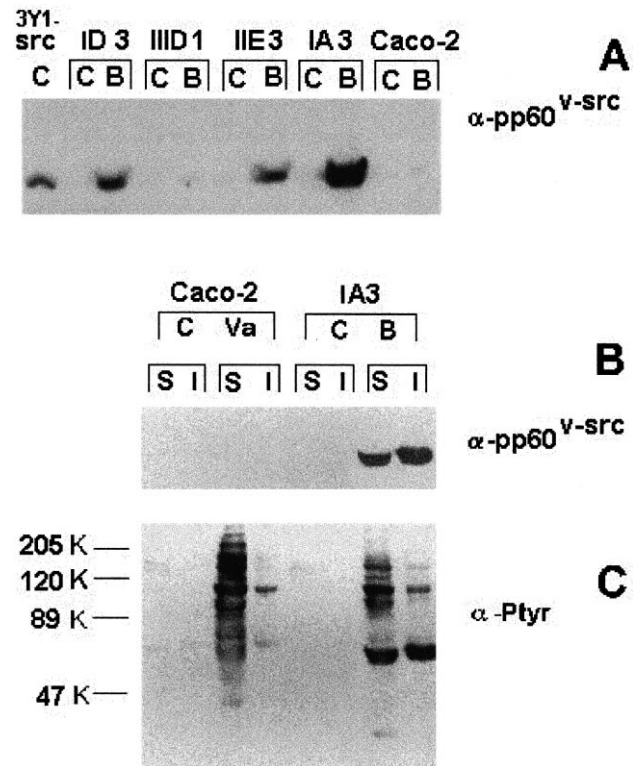


Fig. 1. Expression of pp60v-src by Caco-2 clones. Control Caco-2 cells or different clones were grown until day 5 after confluence; cells were treated with  $\text{VaO}_4\text{Na}_3$  (0.5 mM) (Va) for 6 h or butyrate (5 mM) (B) for 15 h. Total cell extracts (panel A) or Triton-soluble (S) and insoluble (I) fractions (panels B and C) were prepared and analysed by SDS-PAGE (10% polyacrylamide) and Western blot with antibodies EC-10, specific for the viral pp60src (A,B), or anti-Ptyr 4G10 (C). Total extracts of 3Y1 fibroblasts transformed with v-src oncogene were used as positive control (A). No changes in the Ptyr content of control Caco-2 or IIID1 cells were detected when treated with butyrate (not shown). The molecular mass of prestained markers (from top to bottom,  $\beta$ -galactosidase, myosin, bovine seroalbumin and ovalbumin) (BioRad) is indicated.

EDTA) supplemented with 10  $\mu\text{g}/\text{ml}$  aprotinin, 20  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM PMSF and 0.5 mM  $\text{VaO}_4\text{Na}_3$ . Lysates were centrifuged at 13000 rpm in a microfuge for 5 min at 4°C and supernatants were aliquoted and stored at -40°C until use. To analyse tyrosine phosphorylation of proteins, 400  $\mu\text{g}$  of extract were incubated with 5  $\mu\text{g}$  of mAb 4G10 in a final volume of 1 ml for 12 h at 4°C; immunocomplexes were collected adding protein A-agarose for a further hour and spinning in a microfuge. Pellets were extensively washed in RIPA buffer plus inhibitors and solubilised in Laemmli's

sample buffer [6]. After electrophoresis, the presence of p120-cat or  $\beta$ -catenin was determined blotting with specific antibodies.

### 2.8. Other methods

The preparation of Triton-soluble and -insoluble fractions was performed exactly as described [6]. Protein concentration was determined with the BioRad (DC) assay.

## 3. Results

To characterise the regulation of TJ in Caco-2

cells, this cell line was transfected with a plasmid containing the v-src gene under the control of the CMV early promoter. Expression of this oncogene has been reported to alter TJ and AJ in MDCK cells [3]. Several clones were obtained which, after confluence, expressed pp60v-src only when butyrate was added to the culture medium (Fig. 1A). This compound has been shown to boost transcription of recombinant plasmids in mammalian cells [16] and has been used to induce expression of transfected genes in epithelial cells [17–19]. Addition of sodium butyrate (5 mM) to clones IA3, ID3 or IIE3 for 15 h induced a high expression of pp60-vsrc; without this treatment the kinase was undetectable in these clones (Fig. 1A,B). Levels of expression of this

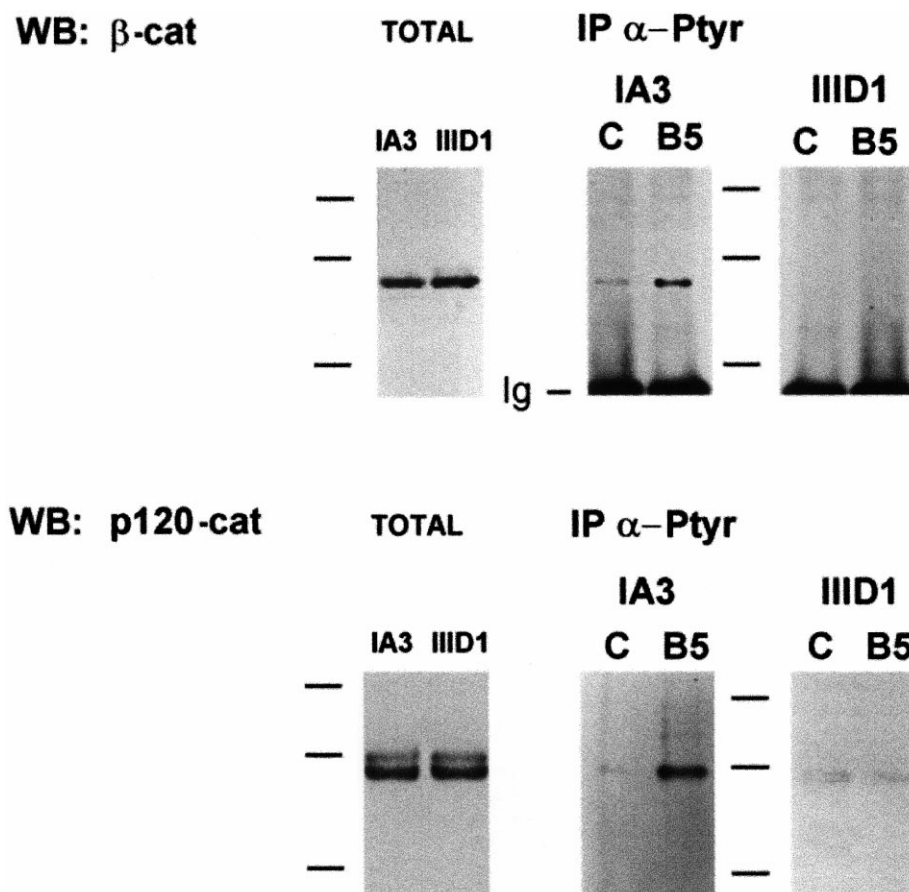


Fig. 2. Tyrosine phosphorylation of catenins after expression of pp60v-src. Cells were grown in complete medium (DME plus 20% FBS) until day 5 after confluence; at this time total cell extracts were prepared from clones IA3 or IIID1 which had been incubated in the absence (C) or presence of butyrate (5 mM for the last 15 h) (B5). Extracts were immunoprecipitated with the anti-phosphotyrosine antibody 4G10, immunocomplexes were subjected to SDS-PAGE and analysed by Western blot with antibodies anti- $\beta$ -catenin or anti-p120-cat. As control, total cell extracts were analysed with these two mAbs. The bars indicate the migration of the prestained molecular mass markers: from top to bottom, myosin (205 kDa),  $\beta$ -galactosidase (120 kDa) and BSA (89 kDa).

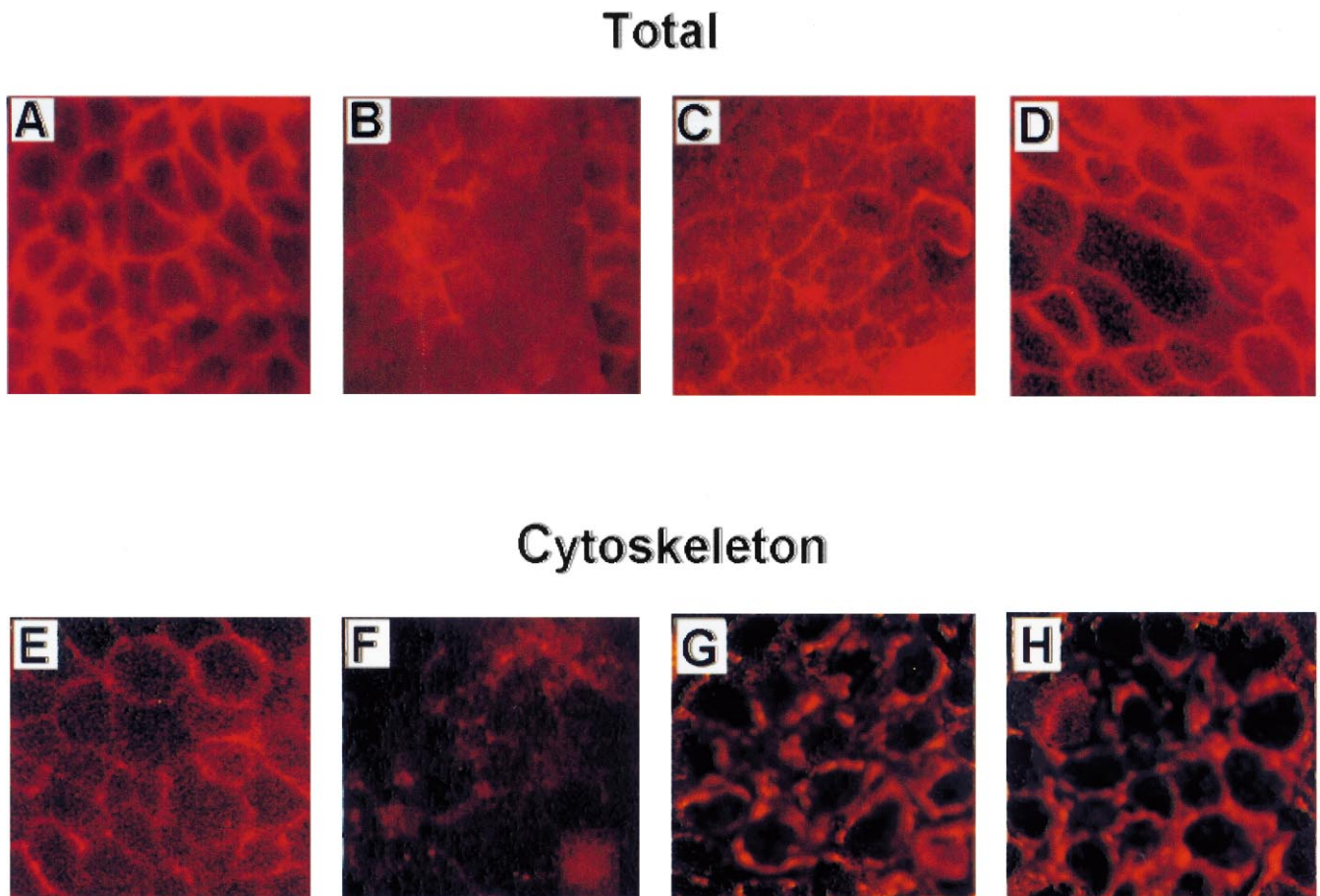


Fig. 3. Expression of pp60v-src alters the distribution of E-cadherin in postconfluent Caco-2 cells. E-Cadherin was analysed by immunofluorescence in 5 days-postconfluent IA3 (right panels: A–B,E–F) or III D1 (left panels: C–D,G–H) cells incubated without further additions (A,C,E,G) or in the presence of butyrate (5 mM for 15 h) (B,D,F,H). Cells were immediately fixated in *p*-formaldehyde (A–D) or incubated with CSK buffer before fixation (E–H) and processed for E-cadherin staining; thus the levels of total (A–D) or cytoskeleton-associated (E–H) E-cadherin are shown. The experiment was repeated twice with similar results. Photographs were taken at 1000 $\times$  magnification.

tyrosine kinase were greater in clone IA3 than those detected in 3Y1 fibroblasts transformed with this oncogene (Fig. 1A). Addition of this compound to clone IA3 up to 24 h did not affect cell viability; butyrate could be removed and the cell monolayer remained intact up to 7 days. No pp60-vsrc expression was observed in the presence of butyrate in untransfected Caco-2 cells or in a transfected clone, IIID1, which was used as a control in our experiments (Fig. 1A). As expected for the active form of this kinase [20], a significant part of pp60v-src was associated to the cytoskeleton in IA3 cells (Fig. 1B). An increase in the P<sub>tyr</sub> content of cellular proteins was detected concomitantly to the expression of pp60v-src in the positive clones, demonstrating that

the synthesised pp60v-src was functional (Fig. 1C). Although the pattern was different, the extent of tyrosine phosphorylation of proteins in IA3 cells expressing pp60v-src was comparable to that obtained after incubation of control cells with  $\text{VnO}_4\text{Na}_3$ , a commonly used phosphotyrosine phosphatase inhibitor.

Two pp60v-src substrates,  $\beta$ -catenin and p120-cat, are involved in the regulation of intercellular contacts [21]. Increased tyrosine phosphorylation of these two proteins correlates with loss of function of E-cadherin and disassembly of AJ [22]. The phosphorylation of these proteins was analysed in IA3 cells at day 5 after confluence, a time when they have already formed a well-polarised impermeable

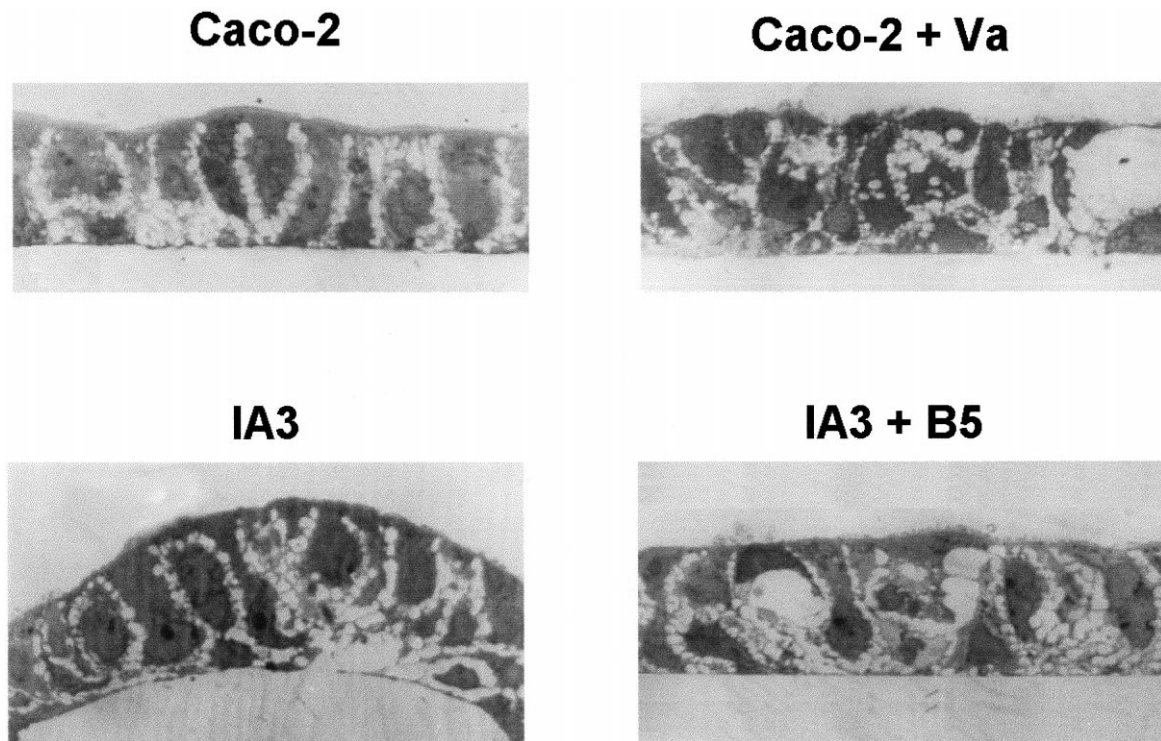


Fig. 4. Semithin sections of postconfluent cultures of Caco-2 or IA3 cells. Day 5 postconfluent Caco-2 or IA3 cells were incubated with  $\text{VaO}_4\text{Na}_3$  (0.5 mM for 6 h) (Va) or butyrate (5 mM for 15 h) (B5). The figure shows representative micrographs of semithin sections taken at  $1000\times$  magnification.

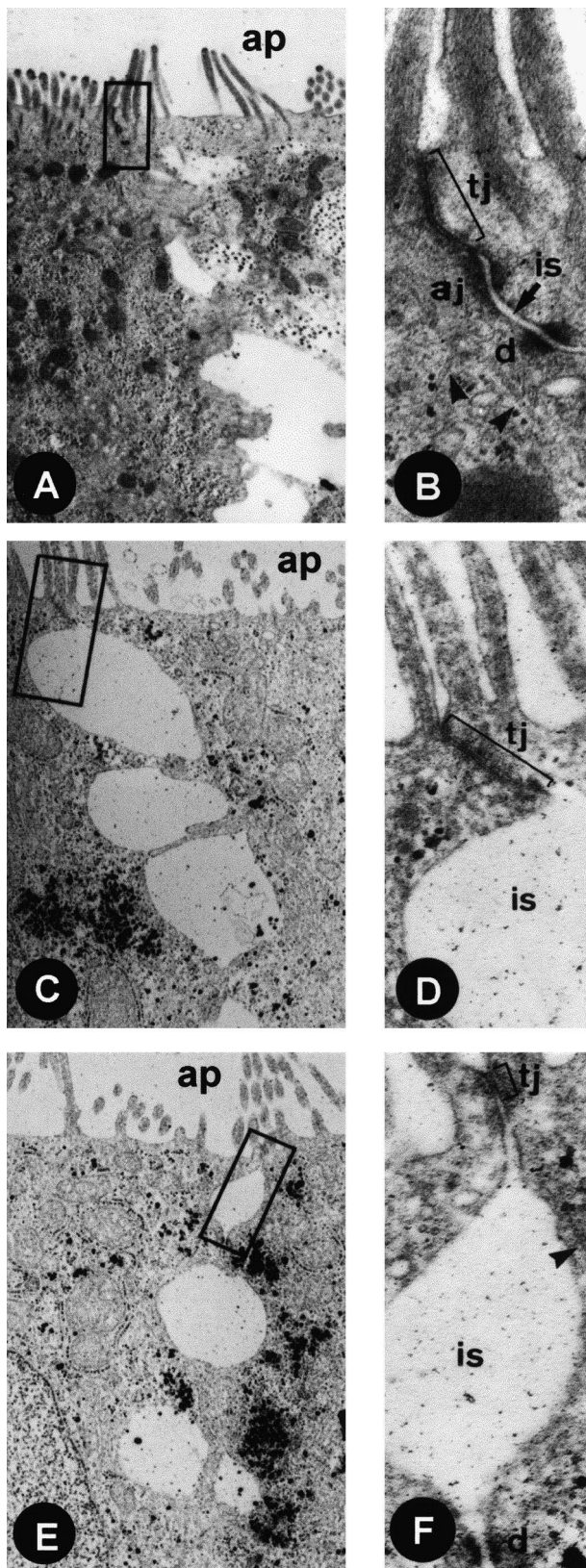
cell monolayer (see Section 1). Induction of pp60v-src expression in these cells increased the P<sub>Tyr</sub> content of  $\beta$ -catenin and p120-cat (Fig. 2). No change in the phosphorylation of these two proteins were observed after addition of butyrate to control Caco-2 (not shown) or IID1 cells, a clone that did not synthesise pp60v-src in these conditions (Fig. 2).

The presence of functional E-cadherin and AJ was also determined in postconfluent cells. E-cadherin was detected mainly in the membrane of IA3 (Fig. 3A) or IID1 cells (Fig. 3C). Expression of pp60v-src by addition of butyrate to IA3 cells induced a redistribution of E-cadherin; although this protein was still present in the cell borders, it was mostly detected, diffusely distributed, throughout the cytosol (Fig. 3B). On the contrary, butyrate did not modify E-cadherin staining in IID1 cells (Fig. 3D). The correct function of E-cadherin requires its association to the actin cytoskeleton, which is reflected by the insolubility of this protein in non-ionic detergents as Triton X-100. Therefore, the presence of E-cadherin was analysed in the same cells, treated with this detergent prior to fixation (Fig. 3E–H). The amount

of detergent-resistant E-cadherin was markedly diminished respect to control IA3 cells (Fig. 3E) after expression of pp60v-src (Fig. 3F). Addition of butyrate to IID1 did not modify the amount of cytoskeleton-associated E-cadherin (Fig. 3G,H). Similar results to those shown with clone IA3 were obtained when the experiment was performed with ID3 cells, that also express pp60v-src after addition of butyrate (not shown).

The existence of morphological alterations in cell junctions was investigated by alternative methods. First, semithin sections from Caco-2 cells or clone IA3 were examined. After confluence, both cell lines form a monolayer of cells showing cell junctions especially in the upper part of the lateral membrane. In the rest of the lateral surface, cells interconnect through membrane extensions but do not show extensive areas of membrane apposition (Fig. 4). Only small differences were detected in IA3 cells when expression of pp60v-src was induced with butyrate: the continuity of the sub-apical interactions was evident and the number of extensions was not substantially altered (Fig. 4). As expected, addition of  $\text{Ca}^{2+}$ -che-





lant agent EGTA totally disorganised the cell monolayer (not shown).

The cells were analysed to a greater detail by transmission electron microscopy. Control IA3 cells, as well as untreated Caco-2, showed the presence of TJ, AJ and desmosomes in the upper part of the lateral membrane (Fig. 5). Intracellular spaces were also visible. After induction of pp60v-src expression AJ could no longer be observed; however, TJ and desmosomes were still present (Fig. 5).

It has been generally assumed that integrity of TJ depends on the existence of AJ (see above). To characterise whether the structures observed in the micrographs shown in Fig. 5 were really functional TJ, several experiments were performed. First, permeability of postconfluent Caco-2 cell monolayers was determined; cells were seeded on transwells and flux of mannitol across the monolayer was measured after different stimuli. This parameter is not influenced by changes in membrane conductance, unlike transepithelial resistance [23]. At day 5, TJ were almost completely impermeable to mannitol; the flux of this compound across the cell monolayer was  $2.8 \times 10^2$  cpm/h  $\text{cm}^2$  ( $\pm 0.7$ ), that corresponds to 1.3% of the value obtained in a control transwell, where no cells were plated. Another characteristic that indirectly reflects the presence of TJ is the ability of these cells grown on plastic to originate 'domes'; the formation of these structures is due to the vectorial transport of electrolytes towards the basolateral surface and requires the integrity of TJ. At day 5, Caco-2 cells showed approximately  $10 \pm 3$  domes/ $\text{cm}^2$ . Neither the permeability to mannitol nor the number of cell domes were affected by the expression of pp60v-src in confluent cells (Table 1). Although mannitol fluxes of clones IA3 and IIE3 were higher

←  
Fig. 5. Ultrastructure of postconfluent IA3 cells monolayers. Low-power survey electron micrographs show sections of control IA3 cells (A), IA3 cells treated with butyrate (5 mM) for 15 h (C) or IA3 cells treated with  $\text{VaO}_4\text{Na}_3$  for 6 h (E). Enlargements of the areas marked in A, C and E are presented in B, D and F, respectively; these micrographs show junctional complexes between lateral membranes of adjacent cells. Aj, adherent junction; ap, apical plasma membrane; d, desmosome; is, intercellular space; tj, tight junction. Microtubules can be seen in B and F (arrowheads). Magnifications: A,C,E,  $\times 7000$ ; B,  $\times 45\,000$ ; D,  $\times 36\,000$ ; F,  $\times 40\,000$ .

Table 1

Paracellular permeability of mannitol is insensitive to pp60v-src expression in postconfluent Caco-2 cells

Cell line	Mannitol flux (cpm/h cm <sup>2</sup> ) ( $\times 10^{-2}$ )	Presence of cell domes (domes/cm <sup>2</sup> )
Caco-2	2.8 $\pm$ 0.7	10 $\pm$ 3
Caco-2+butyrate	2.6 $\pm$ 0.4	11 $\pm$ 4
IA3	6.7 $\pm$ 1.2	30 $\pm$ 5
IA3+butyrate	6.3 $\pm$ 1.1	40 $\pm$ 4
IIE3	6.8 $\pm$ 1.0	N.D.
IIE3+butyrate	5.9 $\pm$ 1.1	N.D.

Cells were incubated in DMEM supplemented with 20% FBS for 5 days after confluence on transwells (for mannitol flux) or 3.4-cm diameter culture plates. Butyrate (5 mM) was added to the cells 15 h before performing the assays. Flux of mannitol was estimated measuring the amount of [<sup>14</sup>C]mannitol in the lower chamber after 1, 2 and 4 h of addition of this compound to the upper chamber. The presence of domes was determined by visual inspection of three independent plates using an inverted phase-contrast microscope at 200 $\times$  magnification. The data presented for mannitol flux correspond to the mean  $\pm$  S.D. of three (clones) or five (Caco-2) independent experiments. N.D., not determined.

than in Caco-2 cells, probably as effect of clonal variability [24], they were not increased by addition of concentrations of butyrate that greatly induced the synthesis of pp60v-src (compare Table 1 and Fig. 1). Clone IA3 presented a greater number of cell domes than the unselected Caco-2 population at this time after confluence; expression of pp60v-src did not collapse these domes; on the contrary, their number increased slightly (Table 1). This increase is probably related to the higher glucose utilisation observed in IA3 cells after expression of pp60v-src (not shown); Zweibaum and coworkers have found a similar association between these two parameters when studying 26 clones of Caco-2 cells [24].

Sodium butyrate has been reported to have different effects on intestinal cells [25–28]. To rule out that the resistance of Caco-2 TJ to tyrosine phosphorylation is not due to side effects of butyrate, another

additional strategy was carried out. VaO<sub>4</sub>Na<sub>3</sub> is a widely used tyrosine phosphatase inhibitor that disrupts AJ in several systems [5,6]. As shown in Fig. 1, addition of VaO<sub>4</sub>Na<sub>3</sub> (0.5 mM) for 6 h increase the P<sub>tyr</sub> content of cellular proteins. As described in other systems, both  $\beta$ -catenin and p120-cat were phosphorylated in response to VaO<sub>4</sub>Na<sub>3</sub> (not shown).

In Caco-2 cells, addition of VaO<sub>4</sub>Na<sub>3</sub> disrupted AJ. In semithin sections, VaO<sub>4</sub>Na<sub>3</sub> modified cell morphology; after addition of this compound Caco-2 (Fig. 4) and IA3 cells (not shown) presented a more disorganised appearance showing numerous vacuoles; however, the sub-apical interaction seemed to be maintained. Analysis of the effect of VaO<sub>4</sub>Na<sub>3</sub> by transmission electron microscopy revealed similar results that in the case of pp60v-src expression: AJ were disrupted whereas TJ and desmosomes were not significantly altered (Fig. 5).

Table 2

VaO<sub>4</sub>Na<sub>3</sub> does not affect mannitol permeability of confluent Caco-2 monolayers

Experimental conditions	Mannitol flux (cpm/h cm <sup>2</sup> ) ( $\times 10^2$ )		
	HT-29 M6	HT-29 M3	Caco-2
No additions	10.5 $\pm$ 3.5	9.5 $\pm$ 3.8	2.8 $\pm$ 0.7
+VaO <sub>4</sub> Na <sub>3</sub> (0.25 mM)	55.6 $\pm$ 10.2	65.8 $\pm$ 12.2	3.4 $\pm$ 1.0
+VaO <sub>4</sub> Na <sub>3</sub> (0.5 mM)	N.D.	N.D.	3.5 $\pm$ 0.8
+VaO <sub>4</sub> Na <sub>3</sub> (1 mM)	N.D.	N.D.	23.2 $\pm$ 13.8
+PMA (100 nM)	42.0 $\pm$ 8.1	42.0 $\pm$ 8.1	3.0 $\pm$ 0.5
+EGTA (2.5 mM)	N.D.	N.D.	136.0 $\pm$ 15

Cells were cultured for 5 (Caco-2) or 10 (HT-29 M6 and HT-29 M3) days after confluence as previously described. When indicated, stimuli were added to the cell medium for 6 h and then mannitol flux was estimated as described in Table 1. Values are mean  $\pm$  S.D. of five experiments performed. N.D., not determined.



The effect of  $\text{VaO}_4\text{Na}_3$  was also studied using as reference other intestinal cell lines, HT-29 M6 and HT-29 M3. These two cell lines differentiate to a mucus-secreting and absorptive phenotype, respectively, and has been previously used by our group [14]. Development of impermeant TJ is delayed in these cells respect to Caco-2 cells. A value of permeability corresponding to 5% of the control,  $10.5 \times 10^2$  cpm/h  $\text{cm}^2$  ( $\pm 3.5$ ), was obtained in these cells at days 8–10, whereas a similar value was reached in Caco-2 only after day 2–3 after confluence.  $\text{VaO}_4\text{Na}_3$  affected differently to HT-29 and Caco-2 cells: addition of 0.5 mM  $\text{VaO}_4\text{Na}_3$  to Caco-2 monolayers did not significantly modify mannitol flux (Table 2); however, a marked effect of this compound was detected on day 10 postconfluent HT-29 M6 and HT-29 M3 cells, even at lower concentrations (Table 2). Another well-studied effector of TJ, the phorbol ester PMA [2],[13,14],[29,30] was also used; this compound increased the permeability to mannitol of both HT-29 cells but not of Caco-2 cells (Table 2). Effects of  $\text{VaO}_4\text{Na}_3$  on Caco-2 monolayer permeability were only observed after incubations longer than 24 h (when 0.5 mM  $\text{VaO}_4\text{Na}_3$  was used), or at shorter times when the concentration of this compound was raised above 1 mM (Table 2). However, cell viability was affected in these conditions and the changes might be due to cell death. Similar results were obtained with another tyrosine phosphatase inhibitor, PAO; this compound increased mannitol flux at earlier times but also affected more rapidly cell viability (data not shown). Although the permeability of the Caco-2 monolayer was not altered by  $\text{VaO}_4\text{Na}_3$  or PMA, it was sensitive to calcium depletion; addition of EGTA rapidly increased mannitol flux (Table 2).

The insensitivity of Caco-2 TJ to tyrosine phos-

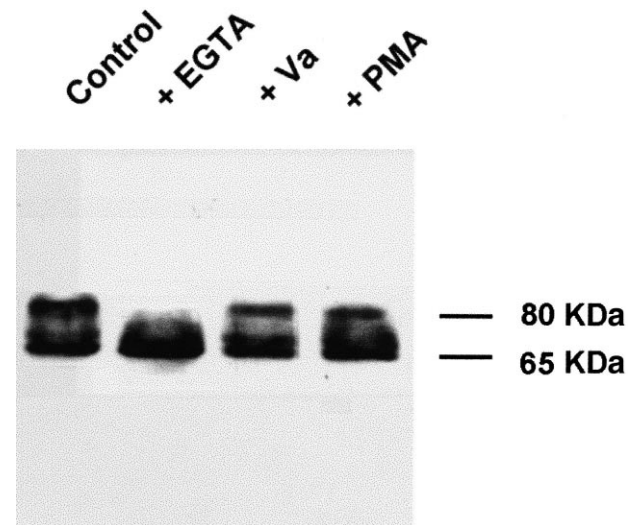


Fig. 6.  $\text{VaO}_4\text{Na}_3$  does not modify occludin hyperphosphorylation. Triton-insoluble fractions were prepared from confluent Caco-2 cells incubated with the indicated additions. EGTA (2.5 mM) was added for 2 h, PMA (100 nM) and  $\text{VaO}_4\text{Na}_3$  (0.5 mM) (Va) for 6 h. The presence of occludin was analysed in these fractions by Western blot. The apparent molecular mass of the two main bands, determined using molecular mass standards, is indicated.

phorylation effectors was confirmed analysing the status of an integral protein present in TJ: occludin [31]. An involvement of occludin phosphorylation in TJ formation has been recently reported [32]; according to these authors functional TJ of MDCK cells only contain highly phosphorylated and detergent-insoluble occludin. Occludin protein present in the Triton-insoluble fraction of Caco-2 cell extracts resolved as several bands, with the highest running with an apparent molecular mass around 80 KDa (Fig. 6). This upper band corresponds to the reported highly phosphorylated form of occludin [32]. As shown for MDCK cells,  $\text{Ca}^{2+}$ -depletion caused the downward shift of the upper bands, in these con-

Table 3

$\text{VaO}_4$  effects on mannitol permeability of Caco-2 monolayers at different days after confluence

Experimental conditions	Mannitol flux (cpm/h $\text{cm}^2$ ) ( $\times 10^2$ )		
	Day 2	Day 4	Day 6
No additions	$9.6 \pm 1.5$	$3.0 \pm 0.7$	$2.7 \pm 0.6$
+ $\text{VaO}_4\text{Na}_3$ (0.25 mM)	$42.5 \pm 8.2$	$4.4 \pm 1.0$	N.D.
+ $\text{VaO}_4\text{Na}_3$ (0.5 mM)	$62.3 \pm 10.8$	$3.7 \pm 0.8$	$3.2 \pm 0.8$

Caco-2 cells were grown in the conditions previously described. Confluence was reached on day 0 and cells were cultured for 2, 4 or 6 additional days. Stimuli were added to the cell medium for 6 h and then mannitol flux was estimated as described in Table 1. Values are mean  $\pm$  S.D. of three experiments performed. N.D., not determined.

ditions only the lower molecular mass band (approx. 65 kDa) was observed. However,  $\text{VaO}_4\text{Na}_3$  or PMA (Fig. 6) did not significantly alter the levels of the 80 kDa hyperphosphorylated form of occludin in Caco-2. These results also suggest that TJ have not been modified by these effectors.

As observed in Table 2, the basal permeability of Caco-2 and HT-29 cell clones differ remarkably. It is generally considered that Caco-2 cells can reach a higher degree of differentiation than HT-29 clones, since they express some markers that HT-29 cells, even those that differentiate to an absorptive phenotype, do not. We consider the possibility that the acquisition of the TJ refractory state was acquired during the process of differentiation. Therefore the response of TJ to  $\text{VaO}_4$  was analysed at different moments after confluence. As previously shown, at day 2 after confluence Caco-2 cell monolayer presented a similar permeability to mannitol than HT29 M6 or M3 cells at later days (Table 2). At this day, Caco-2 TJ were sensitive to addition of relative low amounts of  $\text{VaO}_4$  (Table 3). The increase in mannitol flux was not significantly different than those obtained in HT-29 M3 and M6 cells (compare Table 2 and Table 3). At later days after confluence, the response to  $\text{VaO}_4\text{Na}_3$  was lost; mannitol permeability of day 4 Caco-2 monolayer was not sensitive to this compound (Table 3).

#### 4. Discussion

Regulation of intercellular contacts has been a matter of research since in 1981 when Ojakian described that the phorbol ester PMA disrupted MDCK intercellular adhesion [2]. Since then, other conditions that affect this parameter have been reported: depletion of  $\text{Ca}^{2+}$  or augmentations in tyrosine phosphorylation by addition of  $\text{VaO}_4\text{Na}_3$  or expression of pp60v-src [3,4]. In general terms, in most of the cell systems studied all these conditions affect both types of junctions since disruption of AJ and TJ was normally observed. Therefore is still a matter of controversy if both junctions are co-ordinately regulated or, in other words, if existence of TJ always requires the maintenance of AJ.

The regulation of TJ by pp60v-src has also been a topic of discussion. Initial experiments performed by

Warren and Nelson demonstrated that MDCK cells transfected with low levels of this oncogene, that did not show evident signs of neoplastic transformation, were able to form TJ and desmosomes, but not AJ [33]. Later on, as mentioned above, other authors showed the disappearance of all types of junctions after conditional expression of pp60v-src in the same cell line [3]. This discrepancy has been explained on the basis of the higher levels of expression obtained in the latter experiments, although a careful comparison was not performed, nor were these experiments extended to other cell lines. In addition to the results of Warren and Nelson, very few other experiments have shown a differential regulation of both TJ and AJ. Only Citi and co-workers have reported that AJ disruption caused by depletion of  $\text{Ca}^{2+}$  from the cell medium is not coupled to TJ dissociation if H7 protein kinase inhibitor is added to the cells [34]. This same inhibitor also blocks TJ assembly induced after switching the cells from low  $\text{Ca}^{2+}$  to high  $\text{Ca}^{2+}$  culture medium [35]. The effects of this inhibitor have been explained by suggesting that TJ assembly/disassembly may be dependent on an H7-sensitive kinase that regulates actomyosin-dependent motility [35]. Furthermore, the importance of the modifications of the actin cytoskeleton in the regulation of TJ is consistent with experiments of expression of pp60v-src in MDCK cells. When the level of expression of this oncogene was low, and morphological transformation was not observed, MDCK cells presented disrupted AJ, but retained TJ and desmosomes [33]. However, when high levels of this protein kinase were induced in the same cells, causing morphological transformation, both TJ and AJ were dissociated [3]. In our experiments, neither expression of high levels of pp60v-src nor addition of  $\text{VaO}_4\text{Na}_3$  altered TJ or induced massive cytoskeleton changes in Caco-2 cells; only EGTA had this effect. All these experiments are compatible with a model that suggests that TJ are opened by factors as PMA, EGTA or other factors that stimulate tyrosine phosphorylation, by way of their effect on the actin cytoskeleton, in particular on the actin belt present in AJ. Contraction of this belt might affect TJ permeability and structure. Therefore, TJ disruption would not be a direct consequence of pp60v-src levels but of the ability of this protein kinase to alter actin cytoskeleton. It is possible that this alteration requires a

still unidentified protein, perhaps the H7-sensitive kinase proposed by Citi and Denisenko as involved in the regulation of actin polymerisation, that could be absent or inactive in postconfluent Caco-2 cells.

Our results also indicate that Caco-2 cells are specially resistant to agents that affect tyrosine phosphorylation of proteins as expression of v-src or addition of  $\text{VaO}_4\text{Na}_3$ . The levels of pp60v-src obtained in the clones, especially in IA3 cells, were even higher than those present in 3Y1 fibroblasts transformed with this oncogene and the protein was functional, as demonstrated by the high levels of tyrosine phosphorylation detected in IA3 cells. Even in these conditions, morphological changes were not observed. Addition of concentrations of the PTyr phosphatase inhibitor  $\text{VaO}_4\text{Na}_3$  as high as 0.5 mM for 6 h did not affect either the morphology of postconfluent Caco-2 cells, whereas other cells, for instance HT-29 M6, were sensitive to lower concentrations. However, our results do not mean that Caco-2 cells are totally incapable of responding to this type of stimuli. Other researchers [36] have reported effects of  $\text{VaO}_4\text{Na}_3$  and other inhibitors of PTyr phosphatases on Caco-2 monolayer permeability. We observed that elevated concentrations of  $\text{VaO}_4\text{Na}_3$  (1 mM or higher) also increased mannitol permeability, at the same time that induced marked changes in cell morphology. Moreover, our observations indicate that Caco-2 cells at early moments after confluence, when they show mannitol permeabilities similar to those observed in HT-29 M6 cells, present a significant response to  $\text{VaO}_4\text{Na}_3$ . These observations might indicate that uncoupling of TJ from AJ is the result of maturation of intestinal cells. In any case, the results here present evidence that Caco-2 cells represent an interesting work model to study the specific regulation of junctional complexes.

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